MONTEVERDE, MATTHEW R, M.S. Evaluation of Factors Controlling Methanogens and Methane-Oxidizing Bacteria on Hydropsychid Caddisfly Retreats. (2015)
Directed by Dr. Anne E. Hershey. 47 pp.

Biogenic methane is produced in aquatic sediments by methanogenic Archaea, and captured by methane-oxidizing bacteria (MOB), thereby cycling methane into food webs. Preliminary work has shown that net-spinning caddisfly (Trichoptera: Hydropsychidae) retreats and nets support methanogens and MOB, which are consumed by hydropsychids. We evaluated whether these microbes are incidental on retreats and nets (captured during filter feeding), or if retreat and net microhabitats facilitate their growth. Field data showed that methanogens in hydropsychid foreguts and on retreats were positively associated with seston and sediment methanogen populations, but varied with genus, while net methanogens were also associated with seston but not sediment. *Hydropsyche* nets and retreats were more associated with seston and *Cheumatopsyche* retreats were more associated with sediment. For MOB, *Hydropsyche* retreat populations also were more strongly associated with seston MOB, while *Cheumatopsyche* MOB retreat populations were more closely associated with sediment MOB. But, net MOB populations were not associated with either seston or sediment. These differences may reflect differences in retreat construction and stream microhabitat. In a lab experiment, hydropsychid foregut and net MOB densities were highest in the experimental treatment containing sediment plus methane, and lowest in the treatment with no sediment and equilibrium methane concentration. These results suggest that nets facilitate MOB growth, but that a sediment source may be needed to establish or maintain MOB populations on retreats. It also suggests that MOB are responding to a local methane

source produced by methanogens on nets and retreats which while initially collected passively, reflect patterns of microbial growth and not microbial patterns found in streams.

EVALUATION OF FACTORS CONTROLLING METHANOGENS AND METHANE-OXIDIZING BACTERIA ON HYDROPSYCHID CADDISFLY RETREATS

by

Matthew R. Monteverde

A Thesis Submitted to
the Faculty of The Graduate School at
The University of North Carolina at Greensboro
in Partial Fulfillment
of the Requirements for the Degree
Master of Science

Greensboro
2015

Approved by

_____
Committee Chair

To all my friends and family who have patiently supported and pushed me to achieve more.

APPROVAL PAGE

This thesis has been approved by the following committee of the Faculty of The Graduate School at The University of North Carolina at Greensboro.

Committee Chair _____

Anne E. Hershey

Committee Members _____

Parke A. Rublee

_____

Martin Tsz-Ki Tsui

_____

Date of Acceptance by Committee

_____

Date of Final Oral Examination

ACKNOWLEDGEMENTS

I would like to thank my advisor, Dr. Anne E. Hershey, and committee members Dr. Parke A. Rublee and Dr. Martin Tsz-Ki Tsui for their guidance and support. I would also like to thank Laura Fondario Grubbs and McKenzie Smith for their PCR assistance, and Kimber Corson and Kirsten Trowbridge for help with sample collecting. Funding support for this research project was provided by the UNCG Department of Biology, Julia Taylor Morton Endowment, and the W. John O'Brien Award.

TABLE OF CONTENTS

Page

LIST OF TABLES

Page

LIST OF FIGURES

Page

CHAPTER I

INTRODUCTION

Methane is the most abundant hydrocarbon in our atmosphere (Caldwell et al. 2008). It comes from a variety of sources, such as fossil fuel mining, biofuel combustion and methanogenesis occurring in ruminants, humans, manure, solid waste, waste water and rice fields. It is also produced through methanogenesis in soils and aquatic sediments. Since 1750, the concentration of methane in the atmosphere has increased by 150% (Houghton, 2001). With over 20 times the radiative effectiveness per molecule of carbon dioxide, it ranks second in importance as a greenhouse trace gas, and is a well-studied chemical compound (Caldwell et al. 2008, Shindell et al. 2009).

In aquatic habitats, most methane is produced and concentrated in the sediment, where it eventually diffuses upward and is released through ebullition into the water column or converted to CO_2 by methanotrophs before being emitted to the atmosphere (Whalen 2005). Biogenic methane is produced through methanogenesis. Methanogenesis is the production of methane as a product of methanogen respiration under anoxic conditions (Whalen 2005). Methanogens are Archaea normally found in anaerobic aquatic environments, digestive tracts of animals and the Earth's crust. There are two major pathways of methanogenesis, acetoclastic and hydrogenotrophic. In the acetoclastic pathway, acetate is broken down into methane and carbon dioxide. In the hydrogenotrophic pathway, carbon dioxide (terminal electron acceptor) and hydrogen

combine to form methane and water. Methanogenesis is an important microbial metabolic process and is the last step in the anaerobic decomposition of organic compounds in the carbon cycle.

Methane is not only produced in aquatic habitats, but can also be metabolized there. Methane-oxidizing bacteria (MOB) are bacteria that are able to directly metabolize methane (Hanson and Hanson, 1996). They are commonly found in areas where methane is produced such as soil, landfills, rice paddies and other aquatic habitats such as marshes, oceans, lakes and streams, and often occur in close proximity to methanogens (Hanson and Hanson, 1996). In streams they can occur in either the water column or sediment while also occurring in close proximity with methanogens (Buriankova et al. 2012). MOB have been shown to reduce the amount of methane released into the atmosphere (Valentine and Reeburgh 2000). In wetlands, the amount of methane oxidized is 20 to 40% of what is produced anaerobically (Whalen 2005). In the environment, MOB use methane derived carbon (MDC) as an energy or food source, which may then be used by macroinvertebrates communities (Deines et al. 2007). Through isotope and DNA analysis MDC has been shown to potentially be an important food source in lake and river food webs (Gentzel et al. 2012, Jones and Grey 2011).

Net-spinning caddisflies (Trichoptera: Hydropsychidae) are one of the most important and commonly found macroinvertebrates in streams. They have been shown to impact food webs and contribute to energy flow in streams and lake outlets (Brown et al. 2005, Wallace and Merritt 1980). Hydropsychid caddisflies can be found in high

densities with high production and in temperate environments where large amounts of seston are exported downstream from impoundments or other point source pollutants such as waste water treatment plants (Huryn and Wallace 2000, Mackay and Waters 1986, Parker and Voshell 1983, Parker and Voshell 1982). Hydropsychid caddisflies are also one of the most pollution tolerant families of caddisflies and are often the dominant macroinvertebrate group in disturbed streams (Alexander and Smock 2005, Houghtona 2004).

Hydropsychid caddisflies filter feed by spinning silk nets that capture suspended particulate organic matter from stream water (Wallace et al. 1977). Their nets are usually rectangular and suggestive of tennis racket strings when viewed under a microscope. The size and structure of these nets differ based on species, instar, water temperature and water velocity (Loudon and Alstad 1992). Although it has been shown that hydropsychid caddisflies are important in the cycling of particulate organic matter and contribute greatly to the energy flow in aquatic streams (Brown et al. 2005), their role in the cycling of other forms of biogenic carbon, such as methane, is poorly known.

In addition to consuming particulate organic matter (POM), including small invertebrates that are captured on their nets, hydropsychid caddisflies have been shown to consume methanogens and MOB (Hershey unpublished data). The assimilation of MDC in the diets of macroinvertebrates has been well studied in marine environments (Hanson and Hanson, 1996) and for lentic chironomid larvae (Deines et al. 2007, Eller et al. 2007, Gentzel et al. 2012, Hershey et al. 2006, Hershey et al. 2005). However, it has not been

well studied in many other invertebrates, including hydropsychid caddisflies, or in streams.

Preliminary collections and DNA analysis has shown that hydropsychid retreats support methanogens and MOB (Hershey unpublished data). Some chironomids appear to exhibit "microbial gardening" of MOB on their tubes (Eller et al. 2005, Deines et al. 2007, Jones et al. 2008, Gentzel et al. 2012). A similar behavior could explain the presence of high concentrations of methanogen and MOB on hydropsychid nets and retreats. A study conducted on Psychomyiid caddisflies in oligotrophic and eutrophic lakes found that their retreats were fairly ^{15}N depleted relative to their larval food source, but only slightly depleted in ^{13}C (Ings et al. 2010). The author suggests the possibility that Psychomyiid caddisflies garden their retreats for algae and other microorganisms. Algal gardening has also been observed in grazing Hydroptilid caddisflies (Hart 1985). These caddisflies have been shown to have had a large effect on maintaining the species composition and local abundance of the algal communities that they graze (Hart 1985). A study conducted on *Agapetus* (family: Glossosomatidae) and *Silo* (family: Goeridae) caddisflies in chalk streams found that up to 30% of their carbon was derived from methane sources (Trimmer et al. 2009). Microbial gardening for methanogens and MOB has not been described for hydropsychid caddisflies.

Methanogens and MOB have not been well studied in hydropsychid caddisfly nets and retreats, and it is unknown whether these methanogens and MOB are incidental on nets and retreats (captured during filter feeding), or if the retreat microhabitat

facilitates their growth and leads to increased consumption of these microbes by hydropsychid larvae, which would represent a form of gardening. This study utilized patterns of microbial abundance under field conditions and a laboratory experiment to evaluate whether methanogen and MOB abundance on retreats, nets, and in foreguts was an incidental result of filter feeding or whether these MOB were actively growing on nets in response to local methane supply and subsequently harvested by larvae.

Objectives and Hypotheses

Objective 1: To characterize the methanogen and MOB populations in stream sediments and seston, and compare these to methanogen and MOB patterns on retreats, nets, and foregut contents of two genera of hydropsychid caddisflies from two different Piedmont streams. Results of these comparative studies were used to evaluate whether patterns of abundance of microbes found on retreats and nets reflected patterns in the environment or a whether the patterns were consistent with growth of these microbes on the net and retreat microhabitats.

I hypothesized that the methanogen and MOB communities on the nets and retreats of hydropsychid genera found in each stream reflected taxonomic and stream specific differences in retreat and net microhabitats that were consistent with growth in the microhabitat, but not simply patterns of microbes found in the environment.

Objective 2: To determine if the microhabitat of hydropsychid retreats stimulated the growth of MOB in response to local availability of methane.

I hypothesized that MOB grow on caddisfly nets, facilitated by the microhabitats formed on the nets and/or retreats and local methane supply. I expected to see a higher concentration of MOB on nets and in foreguts in artificial stream microcosms with supplemental methane. I also expected to see a higher concentration of MOB in microcosms fed from North Buffalo Creek water and sediment compared to those fed from Reedy Fork water and sediment reservoir.

CHAPTER II

MATERIALS AND METHODS

Sampling Sites

Two 4th order Piedmont North Carolina streams were chosen for sampling (Fig. 1). North Buffalo Creek (NBC) is an urban stream that drains and receives treated wastewater from northern Greensboro, NC, a city of about 279,000 people (2013 population, Greensboro Planning Department, 2015). NBC was sampled downstream of Rankin Mill Road, based on easy access, abundant hydropsychid caddisflies, and proximity to a USGS stream gauge. Average discharge in NBC during sampled days was 0.57 m^3/s (USGS 2015). The site is about 5 kilometers downstream from the NBC wastewater treatment plant (WWTP). Reedy Fork, sampled in Northeast Park, is a forested stream north of Greensboro that is influenced by low density residential development with forested buffers and recreational use from the park. Average discharge in Reedy Fork during sampled days was 1.05 m^3/s (USGS 2015).

Sample Collection

Objective one

Methane samples (n = 5) were collected using a 10 ml syringe. For each sample, 8 ml of stream water was injected into a 23 ml sealed glass serum vial which had been pre-evacuated, filled with N_2, and treated with 0.1 ml of 1M HCl to arrest microbial

activity. NBC and Reedy Fork were sampled seasonally (Fall: 11/09/2012, Winter: 3/8/2013, Spring: 6/5/2013, and Summer: 9/20/2013) at or near baseflow to quantify methanogen and MOB abundances in seston, sediment, hydropsychid retreats and nets, and hydropsychid foreguts. Hydropsychids were from the genera *Hydropsyche* in NBC and *Cheumatopsyche* in Reedy Fork. Water for seston samples was collected in 4 gallon amber cubicontainers and transported to the University of North Carolina at Greensboro (UNCG) to be immediately filtered. Sediment samples (1.5 ml) were directly collected from the sediment surface of each stream using micro-centrifuge tubes, and introduced into pre-weighed 15 ml Falcon tubes filled with 2 ml of cetyltrimethylammonium bromide (CTAB) buffer (n = 5 per stream). Caddisflies, retreats and net samples were placed into 120 ml Nalgene bottles filled with a 95% ethanol, then stored until they were processed.

Objective two

An artificial stream experiment was conducted near the UNCG greenhouses from July 10th, 2013, through Sep 29th, 2013, in order to determine if the microhabitat of hydropsychid nets stimulates the growth of MOB, or if MOB on nets reflects methane availability in the water. The artificial streams required an area with good ventilation because methane gas was bubbled into some of the streams. Six 38 L plastic containers served as reservoirs to provide water sources for 12 artificial stream channels. Each reservoir fed two cylindrical artificial stream microcosms using submersible pumps, for a total of 12 microcosms.

Two of the reservoirs contained 20 cm of sediment and 19 L water, one from each of the two study streams. The presence of sediment was intended to simulate continuous source populations for MOB that could be entrained into nets. Methane was bubbled into the microcosms to augment methane concentration in the artificial streams. The remaining four reservoirs each contained about 38 L of water from either NBC or Reedy Fork (two in each). Artificial streams fed by one reservoir containing water from each stream were bubbled with methane, similar to those containing sediment, while the artificial streams fed from the other reservoir from each stream were not augmented with methane (Fig. 2).

After the reservoirs were setup, caddisflies were hand collected from both streams and placed into 120 ml bottles for transportation to the artificial stream site. Coarse sediment, rocks, and woody debris were also collected to provide retreat construction materials in each artificial stream for the caddisflies to use. Prior to introduction into artificial streams, larvae were acclimated to the reservoir temperature of 25 °C by floating collection bottles in the reservoir. A trial consisted of introduction of caddisflies into an artificial stream microcosm for 48 hours. Since the experiment focused on microcosm conditions rather than taxonomic differences, both genera were introduced into each microcosm. After 48 hours, methane samples were taken and the caddisflies and their nets and retreats were collected and placed into ethanol until they were ready to be processed. The artificial stream experiment continued until twelve trials were conducted for each of the three treatments.

Sample Processing

Gas from methane vials was later analyzed and quantified for methane (ppm) using gas chromatography. Samples were then corrected for sample volume and headspace volume to obtain total methane concentration (µg/L) (Bretz and Whalen 2014).

Water samples from NBC, Reedy Fork and the artificial streams were filtered immediately upon return to the lab using 25 mm glass fiber filters (GF/F) with 0.7 µm pore size until filters were clogged (~25 ml - 400 ml) to evaluate methanogen (first objective only) and MOB abundance (ng/ml) in seston. Filters were placed into Falcon tubes containing 2 ml of cetyltrimethylammonium bromide (CTAB) buffer for DNA extraction and PCR analysis (Schaefer 1997). For each stream there were five replicates of filtered water samples per sampling period.

Hydropsychids were extracted from their retreats and identified to genus (Merritt and Cummins 2008) with the aid of a dissecting microscope. To determine relative consumption of methanogens and MOB by hydropsychids, foregut contents were dissected from larvae and placed into pre-weighed Falcon tubes which contained 2 ml of CTAB buffer. Approximately 5-10 foreguts from the same hydropsychid genus were used per sample to achieve an approximate weight of 0.1 g foregut material in each Falcon tube. This process was repeated until there were five replicate foregut content samples for each hydropsychid genus on each sampling date.

To determine whether caddisfly nets have differing concentrations of methanogens and MOB than retreats, nets were dissected from retreats for DNA analyses. Following dissection, nets were placed into pre-weighed Falcon tubes with 2 ml of CTAB buffer (5-10 nets in each falcon tube) to achieve a weight of 0.1 g. This process was repeated to obtain five replicate net samples for each hydropsychid genus from each stream. After the nets were removed, caddisfly retreats were isolated from the rest of the collection by removing any remaining invertebrates and miscellaneous debris still attached to the retreats. Retreats were placed in Falcon tubes with 2 ml of CTAB buffer (3-6 retreats in each falcon tube) to achieve a weight of at least 1 g of sample in each falcon tube. This process was repeated to obtain five replicate retreat samples for each hydropsychid genus.

Artificial stream samples were processed in the same manner as described above for the seasonal samples, except that the nets were separated from the retreats after sampling and were pooled for each artificial stream.

DNA Extraction and PCR Analysis

DNA was extracted from the samples using the CTAB extraction method (Schaefer 1997). DNA quantity and purity were determined from 2 µl subsamples of each extraction using a Thermo Scientific Nanodrop Spectrophotometer by pipetting based on the 260/280 wavelength ratio (DNA is generally ~1.8, Thermo Scientific Nanodrop 1000 user's manual, 2008). Samples that deviated above 2.2 or below 1.6 from the 260/280 ratio of 1.8 were resampled then eliminated if they still did not approximate the 1.8 ratio.

Extracted DNA samples were then diluted to 5ng μl^{-1} using TE buffer. The diluted samples were then stored in a refrigerator until analysis.

Real time PCR was performed to quantify the methanogen and MOB DNA in each sample using the Applied Biosystems StepOne real-time PCR System. General methanogen (Wright and Primm 2003) and MOB primers (Costello and Lidstrom 1999) were used to quantify total DNA concentrations (Table 1). Primer pairs Met86/Met1340 and A189/mb661 were used for methanogen and MOB quantitative analysis (Gentzel et al. 2012). The methanogen primer pair targeted 16s rRNA specific to methanogens, while the MOB primer pair targeted the particulate methane monooxygenase (pMMO) gene (Gentzel et al. 2012). Methanogen standards originated from genomic DNA of *Methanosarcina acetivorans* and MOB standards originated from genomic DNA of *Methylococcus capsulatus*. Each run consisted of duplicate negative controls and four concentrations of triplicate standards. Each sample was run in duplicate or triplicate if a wide deviation in Ct value was present between the samples. Each sample reaction consisted of a mixture of: 1µl of extracted DNA, 1µl of the forward primer, 1µl of the reverse primer, 8µl of sterile deionized (DI) water and 10µL of Power Sybr Green PCR master mix. To ensure proper amplification and target binding of DNA, each melt curve was visually examined for comparison to corresponding standards.

Statistical Methods

Statistical analyses for both objectives were run using JMP® statistical software (JMP®, version 10). P-values < 0.05 were considered significantly different for each

analysis. Most datasets were log (x+1) transformed to meet parametric model assumptions.

For the first objective, two-way ANOVA's were performed to evaluate the seasonal and stream differences and interaction of those main effects in methane concentration, and methanogen and MOB abundance in caddisfly foreguts, on retreats and nets, and in sediment and seston. If there were significant seasonal differences, Tukey's post-hoc tests were then run to evaluate those differences.

For the second objective, effects of stream source, treatment and the interaction of stream source and treatment on methane concentration and MOB abundance on hydropsychid nets were evaluated using a two-way ANOVA. If there were significant differences between treatments and stream types, one-way ANOVA's were run with Tukey's post-hoc tests to evaluate which factors were significantly different.

A three-way ANOVA was used to evaluate log transformed foregut MOB abundance for artificial stream treatments (sediment bubbled vs. no sediment bubbled vs. no sediment no methane), stream source (Reedy Fork or NBC) and hydropsychid genus, along with their respective interactions. Significant stream and treatment effects in the three-way ANOVA were followed by a two-way ANOVA evaluating these differences and any interactive effects. If there were significant treatment and stream effects, one-way ANOVA's with Tukey's post-hoc tests were run to evaluate which factors were significantly different.

CHAPTER III

RESULTS

Methane Concentrations at Study Sites

Methane concentrations at study sites varied only slightly across seasons, but there were still some seasonal methane differences. A two-way ANOVA determined that there were significant differences in streams ($p < 0.0001$), seasons ($p < 0.0001$), and interactions between them ($p < 0.0001$, Fig. 3). Average methane concentration in NBC ranged from 1.7 μg/L to 2.5 μg/L with significantly lower concentration in the summer than all other seasons ($p < 0.0001$, Fig. 3). Average methane concentration from Reedy Fork was lower than NBC and ranged from 0.5 μg/L to 1.1 μg/L with significantly higher concentration in the spring than in the summer and fall ($p < 0.0001$, Fig. 3).

Methanogen Abundance at Study Sites

Sampled components for methanogen abundance varied widely based on season and some components differed between streams. A two-way ANOVA found no significant interactions between season and stream for any of the sampled components (Table 2), however foregut methanogens were significantly different between seasons ($p < 0.0001$, Table 2) and hydropsychids in Reedy Fork had significantly higher methanogen abundance in foreguts than those from NBC ($p = 0.0002$, Table 2, Fig. 4A). When evaluated by stream, Reedy Fork, mean foregut methanogen abundance was

highest during fall and lowest during summer. Fall and winter values were significantly higher than spring and summer ($p < 0.0001$, Table 2). In NBC, foregut methanogens were also highest during fall and lowest during summer with fall values significantly higher than all other seasons ($p = 0.0042$). Net mean methanogen abundance differed significantly different between seasons ($p = 0.0001$) and streams, with hydropsychid nets having higher methanogen abundance in NBC than in Reedy Fork ($p = 0.0039$, Fig. 4B). When evaluated by stream, Reedy Fork net methanogen abundance showed no seasonal differences, while net methanogen abundance in NBC was significantly lower in spring than in all other seasons ($p = 0.0001$). Retreat mean methanogen abundance was significantly different by season ($p < 0.0001$) but not genera (Fig. 4E). When evaluated by stream, Reedy Fork was highest in the fall ($p < 0.0001$) and significantly lower in spring and summer, while NBC methanogen mean abundance on retreats was highest in the Fall ($p = 0.0022$) and significantly lower in the other three seasons. Sediment mean methanogen abundance was significantly different by season ($p < 0.0001$) but not between streams (Fig. 4G). When evaluated by stream, Reedy Fork had higher sediment methanogen abundance during the fall and winter ($p < 0.0001$) than during the spring and summer. In NBC, sediment methanogens were significantly more abundant in the fall ($p < 0.0001$) than all other seasons, and significantly more abundant in winter than in spring and summer. Seston mean methanogen abundance was significantly different by season ($p = 0.0088$) but not between streams (Fig. 4I). When evaluated by stream, Reedy Fork showed no significant seasonal differences, while NBC seston methanogen abundance

was significantly higher during winter and fall than during the spring and summer ($p = 0.0085$).

Regression analysis showed that methanogen abundance on hydropsychid retreats was significantly related to methanogen abundance in the seston in NBC ($p = 0.0004$, $R^2 = 0.8127$, Fig. 5A), but not seston in Reedy Fork (Fig. 5B). Methanogen abundance on hydropsychid retreats was also significantly related to methanogen abundance in the sediment in NBC ($p = 0.0002$, $R^2 = 0.8425$, Fig. 5C), but not methanogens in sediments in Reedy Fork (Fig. 5D). Methanogen abundance on hydropsychid nets was significantly related to methanogen abundance in the seston in NBC ($p < 0.002$, $R^2 = 0.5748$, Fig. 5E), but not methanogens in sediments in Reedy Fork (Fig. 5F). Foregut methanogen abundance was significantly related to methanogen abundance in retreats for *Hydropsyche* in NBC ($p = 0.0003$, $R^2 = 0.8677$, Fig. 5G) and *Cheumatopsyche* in Reedy Fork ($p = 0.0008$, $R^2 = 0.5401$, Fig. 5H). However, foregut methanogen abundance was not significantly related to net methanogen abundance for either caddisfly genus (Fig. 5I and 5J).

Methane-Oxidizing Bacteria Abundance at Study Sites

A two-way ANOVA evaluating foregut MOB determined that there were significant differences in season ($p < 0.0001$, Table 2), significantly greater abundance of methanogens in foreguts from NBC than Reedy ($p = 0.0116$, Table 2, Fig. 4B) and significant interactions between seasons and streams ($p = 0.0003$, Table 2). When evaluated by stream, Reedy Fork mean foregut MOB abundance was significantly higher

during spring than in all other seasons, and significantly higher in winter than in summer and fall ($p < 0.0001$, Table 2). In NBC, foregut MOB abundance showed no seasonal differences. MOB abundance on hydropsychid nets were significantly different by season ($p = 0.001$), greater in NBC than Reedy Fork ($p < 0.0001$, Fig. 4D) and showed a significant interaction between seasons and streams ($p = 0.0246$). When evaluated by stream, Reedy Fork also showed no seasonal differences MOB abundance on nets, while in NBC, MOB abundance on nets was significantly higher during the winter than all other seasons ($p = 0.0003$). Retreat mean MOB abundance was significantly different by season ($p < 0.0001$), higher in NBC than Reedy Fork, ($p = 0.0231$, Fig. 4F) and showed a significant interaction between season and stream ($p < 0.0001$). When evaluated by stream, Reedy Fork retreat MOB abundance was significantly higher during fall winter and spring than in the summer ($p = 0.0004$, Table 2), while in NBC, retreat MOB abundance was significantly higher during the spring than all other seasons and lower during summer and winter than in the fall ($p < 0.0001$). Mean MOB abundance in Reedy Fork sediment was significantly different by season ($p < 0.0001$), greater in Reedy Fork than NBC ($p < 0.0001$, Fig. 4H) and showed a significant interaction between season and stream ($p < 0.0001$). When evaluated by stream, sediment MOB abundance in Reedy Fork was significantly higher during winter, fall and spring than in the summer ($p < 0.0001$), while NBC was significantly higher during the winter than all other seasons ($p < 0.0001$). Seston MOB seasonality was significantly different by season ($p < 0.0001$) but not between streams (Fig. 4J). However, the interactions between season and stream were significant ($p = 0.0066$). When evaluated by stream, both streams had significantly

higher seston MOB abundance during spring (Reedy Fork- $p = 0.0281$; NBC- $p = 0.001$) and lower during all other seasons.

Retreat MOB abundance was significantly related to seston in NBC ($p = 0.0004$, $R^2 = 0.637$, Fig. 6A), but not seston in Reedy Fork (Fig. 6B). Retreat MOB abundance was also not significantly related to sediment in NBC (Fig. 6C), but was significantly related to sediment in Reedy Fork ($p = 0.0003$, $R^2 = 0.5935$, Fig. 6D). Net MOB abundance was not significantly related to seston in NBC (Fig. 6E) or sediment in Reedy Fork (Fig. 6F). Foregut MOB abundance was not significantly related to either retreat (Figs. 6G and 6H) or net (Figs. 6I and 6J) abundance for either caddisfly genus.

Methane-Oxidizing Bacteria Responses to Methanogens

Pooled foregut MOB abundance from both genera did not significantly respond to foregut methanogens ($R^2 = 0.0563$, Fig. 7A). Net MOB abundance, however, did significantly respond to net methanogens ($p = 0.0016$, $R^2 = 0.4162$, Fig. 7B). Retreat MOB samples also significantly responded to retreat methanogens ($p = 0.0042$, $R^2 = 0.2835$, Fig. 7C). Sediment MOB samples significantly responded to sediment methanogens ($p = 0.0004$, $R^2 = 0.3875$, Fig. 7D). Seston MOB samples did not significantly respond to seston methanogens ($R^2 = 0.0014$, Fig. 7E).

Artificial Stream Methane Concentration

Artificial stream methane concentrations ranged from 0.3 µg/L to 965.1 µg/L. A two-way ANOVA determined that treatments differences were statistically significant (p

< 0.0001, Fig 8), and there were no significant stream source or interaction effects between stream source and treatment. The bubbled treatments without sediment had the highest methane and were approximately 3 – 5 times higher than sediment bubbled treatments which had an intermediate concentration of methane. Bubbled treatments both had 1–3 orders of magnitude higher methane concentration than the no sediment no methane bubbled treatment.

Artificial Stream Net Methane-Oxidizing Bacteria Abundance

A two-way ANOVA determined that MOB abundance on hydropsychid nets differed significantly between all treatments. The sediment bubbled treatment had the highest MOB abundance and the no sediment no bubbled had the lowest ($p < 0.0001$, Table 3). Furthermore, reservoirs with NBC water and/or sediment had higher net MOB abundance than those with Reedy Fork water and/or sediment ($p = 0.001$, Table 3) and there was a significant interactive effect between stream source and treatment ($p = 0.027$). A subsequent one-way ANOVA followed by Tukey's test showed that NBC bubbled treatments, regardless of the presence of sediments, had significantly higher MOB abundance on caddisfly nets than non-bubbled treatments (Fig. 9, Table 3, $p < 0.0001$). However, Reedy Fork sediment bubbled treatment was significantly higher than both non-sediment treatments ($p < 0.0001$).

Artificial Stream Foregut Methane-Oxidizing Bacteria Abundance

A three-way ANOVA showed that there were significant treatment and stream sources effects on foregut MOB, but no significant effect of genus and no significant

interactive effects between any of the components. Hydropsychids from NBC reservoirs had higher foregut MOB mean abundance than those from Reedy Fork ($p = 0.0124$, Table 3, Fig. 10). All three treatments were significantly different from one another; hydropsychids in the sediment bubbled treatment had the highest foregut MOB abundance and the no sediment no bubbled treatment had the lowest ($p < 0.0001$). A two-way ANOVA determined that even after pooling caddisfly genera (which did not show any significant difference) from our model, patterns of stream ($p = 0.0111$) and treatment differences ($p < 0.0001$) were still the same. One-way ANOVA's for artificial streams from each stream source showed that hydropsychids foregut MOB abundance in NBC artificial streams was not significantly different between the two bubbled treatments, which were both greater than the no sediment no bubbled treatment ($p = 0.004$, Fig. 11, Table 3). For Reedy Fork artificial streams, the sediment bubbled treatment resulted in hydropsychids with significantly higher foregut MOB concentrations than both non-sediment treatments which were not significantly different from one another ($p < 0.0001$, Fig. 11, Table 3).

CHAPTER IV

DISCUSSION

This study was designed to evaluate methanogen and MOB populations in streams and compare them to methanogen and MOB patterns found on hydropsychid retreats, nets and foregut contents in an urban and a reference stream, and evaluate whether MOB populations on retreats, nets and in foreguts were an incidental result of filter feeding or a response to local methane availability. Patterns of methanogen and MOB abundances on hydropsychid nets and retreats reflected differences in caddisfly retreat construction and stream microhabitat and were not consistent with capture of these organisms during filter feeding as the only mechanism. A laboratory experiment showed that MOB growth on retreats and nets was responding to methane availability, although a sediment source also may be important to establish and/or maintain these populations.

Evaluation of Field Patterns in Methane Concentration and Methanogen and MOB Abundance

Higher concentrations of methane in the urban compared to the reference stream could be due to multiple factors, including greater organic matter inputs (Acuña et al. 2007, Meyer 1980) that could stimulate in-stream methanogenesis, and greater delivery of methane from groundwater and upslope riparian and terrestrial zones (Jones and Mulholland 1998), as well as upstream, especially the wastewater treatment plant. Although there were some significant seasonal differences, the magnitude of these

differences was small. Methane in NBC and Reedy Fork are comparable to concentrations in Nine-Mile Creek located in the rural canyons in central Utah (1.2 - 3.2µg/L) (Heilweil et al. 2013) and West Bear Creek, a low order piedmont NC stream (~1 - ~12µg/L) (Heilweil et al. 2014). Overall observed methane concentrations were relatively low when compared to other piedmont streams (5 – 500 µg/L) depending on season (Smith 2013) and lowland chalk rivers in England which ranged between 16 – 100 µg/L (Shelley et al. 2015).

Patterns of methanogen and MOB abundance are likely related to differences in caddisfly retreat and/or net construction and stream microhabitat. Hydropsychids are a diverse family of caddisflies that differ in their retreat and net construction techniques by species (Wallace and Merritt 1980), larval instar (Alstad 1980), temperature (Philipson and Moorhouse 1974), stream velocity (Edington 1968), and substrate and construction materials available in streams (Cudney and Wallace 1980). Thus, both taxonomic and in-stream conditions that control retreat and net construction could have affected methanogen and MOB abundance in retreats and on nets. In this study, *Hydropsyche* in NBC built their retreats using aquatic vegetation on the upper surfaces of rocks, and were directly exposed to the water column. *Cheumatopsyche* built their retreats under rocks using coarse sediment. These differences were reflected in the strong positive relationships between seston and retreat methanogen and MOB abundance for *Hydropsyche* and sediment and retreat methanogens and MOB for *Cheumatopsyche* (Fig. 5 & 6). Thus, where and how hydropsychids build their retreats likely impacts microbial population abundance and availability found on retreats.

Patterns of methanogen and MOB abundance in hydropsychid foreguts reflect sediment and seston availability and growth on hydropsychid retreats and nets. In our field study, foregut methanogens were positively associated with retreat methanogen abundance for both genera (Fig. 5G, H) but not methanogen abundance on nets (Fig. 5I, J). This suggests that hydropsychids are likely consuming more methanogens found in their retreats than on nets. Retreat feeding has not been studied in hydropsychid caddisflies. However grazing case building sericostomatids have been shown to graze on cases as well as on the surrounding substrate (Bergey and Resh 1994) and glossosomatid caddisflies (Cox and Wagner 1989) feed on algae on their cases as well as on the substrate. MOB foregut abundance was not significantly related to either retreat or net abundance for either genus which suggests that MOB, thus examination of these patterns does not provide any insight into hydropsychid feeding on MOB.

Methanogens produce methane that MOB can use as a carbon source during respiration, usually in close proximity to methane production by methanogens (Kajan and Frenzel 1999). The significant regression for MOB response to methanogens on nets (Fig. 7B), retreats (Fig. 7C) and sediment (Fig. 7D) is consistent with a spatially explicit response of MOB to methanogens (Chanton 2005). Not surprisingly, caddisfly foregut contents (Fig. 7A) and seston (Fig. 7E) MOB abundance were not significantly related to methanogens in those respective habitats. Foreguts of most animals, including caddisflies, are anaerobic and would not be expected to support methane oxidation. Methanogenesis occurs in anaerobic habitats, but has not been reported in insect foreguts to our knowledge. The significant relationships between MOB and methanogens on both

nets and retreats (Fig 7B and 7C) are consistent with MOB growth in those respective microhabitats as a response to local production of methane. Such close association of methanogens and MOB has been demonstrated for tubes of larval *Chironomus*.

Experimental Evaluation of MOB Response to Methane

Artificial stream treatments supplemented with bubbled methane but without sediment had on average 3 fold higher methane concentrations than bubbled streams with sediment, while streams without methane bubbled had concentrations that were 50 fold lower than sediment bubbled concentrations (Fig. 8) and were comparable to ambient stream water methane concentrations (Fig. 3). Despite being significantly different, methane concentrations in sediment bubbled and non-sediment bubbled streams still demonstrate concentrations that were on average 50 to 300 fold higher compared to ambient conditions in NBC and Reedy Fork, respectively (Fig. 3). Although much higher than methane concentrations in NBC or Reedy Fork, artificial stream methane concentrations were not unrealistic compared to potential concentrations. Groundwater discharging into a hardwood forest stream in eastern Tennessee was between 90.5 - 736 µg/L which are comparable to our bubbled treatments that ranged between 50 - 450 µg/L (Jones and Mulholland 1998). Local piedmont streams have also been shown to have comparable methane concentrations ranging from 5 – 500 µg/L (Smith 2013).

The overall increase in MOB abundance in the bubbled treatments illustrate that MOB are growing there in response to experimentally increased methane availability in their local environment, although they are also likely captured incidentally during filter

feeding. Furthermore, MOB populations on nets responded quickly to high methane availability, given that larvae were only in the artificial streams for 48 hours. However, as discussed below, these responses also depended on stream source.

MOB abundance on caddisfly nets responded to available methane in the artificial streams, which varied by the stream source, treatment type and net construction. However, MOB abundance on nets was also stream source specific, and varied depending on whether sediment was present or not. Net construction could also factor in MOB abundance because, as discussed above for objective 1, *Cheumatopsyche* in Reedy Fork built their retreats under rocks using sediment, which provides a direct local source of MOB (Chanton 2005). The intermediate MOB response in the bubbled no sediment Reedy Fork treatment compared to the other two treatments (Fig. 9) suggests that entrainment of MOB from the sediments may facilitate establishment of MOB on the nets. Thus, this treatment may have had insufficient time for MOB populations to become established because their abundance was lower initially.

MOB abundance in caddisfly foreguts responded to MOB availability on caddisfly nets which depended on stream source and treatment. Similar stream and treatment MOB abundance patterns between nets (Fig. 9) and foreguts (Fig. 11) illustrate that caddisfly MOB consumption is a direct response to MOB growing on nets and not taxonomic differences in diet. Overall importance of MOB in hydropsychid diets is not known. Given that methane concentrations in NBC and Reedy were low relative to many reports elsewhere, and that MOB in nets, retreats, and foregut contents increase in

response to greater local availability of methane, it is likely that consumption of MOB is universal in hydropsychids, and its overall importance warrants further investigation.

General Discussion and Conclusions

This study examined whether methanogen and MOB associated with hydropsychid nets and retreats are facilitated by the microhabitats provided by those structures, or if they are incidentally captured during filter feeding. Field data suggest that microbial populations found on caddisfly retreats reflected generic differences in retreat construction (using sediment under rock vs. on rock surfaces and using aquatic vegetation) and stream microhabitat (*Cheumatopsyche*: under and between rocks, *Hydropsyche*: on upper surfaces of rocks in direct contact with the water column). Some of the regressions (Fig. 5A, 5C, 5D, 5E & 6A, 6D) are consistent with passive collection of methanogens and MOB onto retreats and nets. However some regressions that would be consistent with passive collection hypothesis were not significant (Fig. 5B, 5F, 6B, 6C, 6E, and 6F), there was considerable unexplained variation of methanogen and MOB concentrations on nets and retreats (Table 2), and elevated MOB abundance from the artificial stream study suggests a direct response to a local methane source. Thus, a more likely conclusion is that these microbes are also growing on the nets and retreats after they collect there passively. Methanogen and MOB captured during filter feeding may provide the conduit for microbial colonization on the nets and retreats. Furthermore, microbes associated with material used to construct retreats would also provide seed microbial populations.

The artificial stream study illustrated that MOB associated with nets respond directly to methane in the water or local retreat environment, and MOB growth on nets results in increased MOB ingestion by hydropsychids. The strong positive relationships between foregut and retreat methanogen abundance found for the field data, combined with the significant response of MOB to methane on nets and foreguts in the artificial stream experiment, strongly suggest that hydropsychids feed on both nets and retreats. The field results also show that MOB are consumed by hydropsychids even when methane concentration in the water is relatively low compared to concentrations typically reported for streams. Such consumption is likely enhanced by growth of the microbes on the nets and/or retreats rather than simple capture. *Chironomus* are believed to garden their tubes to facilitate MOB growth (Eller et al. 2005, Deines et al. 2007, Jones et al. 2008, Gentzel et al. 2012). Gardening of algae has been shown for Hydroptilid caddisflies (Hart 1985), but no such gardening of MOB has been studied for caddisflies. Our data are consistent with this process, but further study would be needed to evaluate it. Further study is also needed to evaluate dietary importance of MOB as well as methanogens, but it is likely that that these microbes are consumed by hydropsychids in all streams. Furthermore, although filter feeding by hydropsychids has received considerable attention, our results suggest that consumption of microbes that are locally grown on nets and retreats also occurs and that MOB on nets respond to changes in methane availability. Thus, more research is needed to evaluate the importance of harvesting locally grown microbes, i.e., gardening behavior, relative to that of filter feeding.

REFERENCES

Acuña, V., Giorgi, A., Muñoz, I., Sabater, F., & Sabater, S. (March 01, 2007). Meteorological and riparian influences on organic matter dynamics in a forested Mediterranean stream. *Journal of the North American Benthological Society, 26,* 1, 54-69.

Alexander, S., & Smock, L. A. (January 01, 2005). Life Histories and Production of Cheumatopsyche analis and Hydropsyche betteni (Trichoptera: Hydropsychidae) in an Urban Virginia Stream. Northeastern Naturalist, 12, 4, 433-446.

Alstad, D. N. (January 01, 1980). Comparative Biology of the Common Utah Hydropsychidae (Trichoptera). American Midland Naturalist, 103, 1, 167.

Bergey, Elizabeth. A., and Resh, Vincent. H. (April 01, 1994). Interactions between a stream caddisfly and the algae on its case: factors affecting algal quantity. *Freshwater Biology, 31,* 2, 153-163.

Bretz, K. A., & Whalen, S. C. (January 28, 2014). Methane cycling dynamics in sediments of Alaskan Arctic Foothill lakes. *Journal of Solid Tumors, 4,* 1, 65-78.

Brown, S. A., Ruxton, G. D., Pickup, R. W., & Humphries, S. (January 01, 2005). Seston capture by Hydropsyche siltalai and the accuracy of capture efficiency estimates.*Freshwater Biology, 50,* 1, 113-126.

Buriankova, I., Brablcova, L., Mach, V., Hyblova, A., Badurova, P., Cupalova, J., Rulik, M., Cap, L. (August 01, 2012). Methanogens and methanotrophs distribution in the hyporheic sediments of a small lowland stream. *Fundamental and Applied Limnology, 181,* 2, 87-102.

Caldwell, S. L., Laidler, J. R., Brewer, E. A., Eberly, J. O., Sandborgh, S. C., & Colwell, F. S. (September 15, 2008). Anaerobic Oxidation of Methane: Mechanisms, Bioenergetics, and the Ecology of Associated Microorganisms. *Environmental Science & Technology, 42,* 18, 6791-6799.

Chanton, J. P. 2005. The effect of gas transport on the isotopic signature of methane in wetlands. Organic Geochemistry 36: 753-768.

Costello, A. M., & Lidstrom, M. E. (January 01, 1999). Molecular characterization of functional and phylogenetic genes from natural populations of methanotrophs in lake sediments. *Applied and Environmental Microbiology, 65,* 11, 5066-74.

Cox, E. J., & Wagner, R. (May 01, 1989). Does *Agapetus fuscipes* cultivate algae in its case?. *Hydrobiologia : the International Journal of Aquatic Sciences, 175*, 2, 117-120.

Cudney, M.D., and J.B. Wallace. 1980. Life cycles, microdistribution, and production dynamics of six species of net-spinning caddisflies in a large southeastern (USA) river. Holarctic Ecology 3:169-182.

Deines P, Bodelier PLE, Eller G. 2007. Methane-derived carbon flows through methane-oxidizing bacteria to higher trophic levels in aquatic systems. Environ Microbiol. 9:1126–1134.Edington, J. M. 1968. Habitat preferences in net-spinning caddis larvae with special reference to the influence of water velocity. - J. Anim. Ecol. 37: 675-692.

Eller G, Deines P, Kruger M. 2007. Possible sources of methane-derived carbon for chironomid larvae. Aquat Microb Ecol. 46:283–293.

Eller G, Deines P, Grey J, Richnow H, Kruger M. 2005. Methane cycling in lake sediments and its influence on chironomid larval δ13C. FEMS Microbiol Ecol. 54:339–350.

Gentzel, T., Hershey, A. E., Rublee, P. A., & Whalen, S. C. (December 01, 2012). Net sediment production of methane, distribution of methanogens and methane-oxidizing bacteria, and utilization of methane-derived carbon in an arctic lake. *Inland Waters, 2*, 2, 77-88.

Greensboro Planning Department (January 01, 2015). *City of Greensboro Growth & Development Trends*. Greensboro Population and Statistics.

Hanson RS, Hanson TE. 1996. Methanotrophic bacteria. Microbiol Rev. 60:439–471.

Hart, D. D. (March 01, 1985). Grazing Insects Mediate Algal Interactions in a Stream Benthic Community. *Oikos, 44*, 1, 40-46.

Heilweil, V., Stolp, B., & Susong, D. (May 23, 2014). Development of stream-based monitoring methods for assessing effects of natural gas development on water resources. http://ut.water.usgs.gov/projects/methanestream/ Retrieved April 7, 2015.

Heilweil, V. M., Stolp, B. J., Kimball, B. A., Susong, D. D., Marston, T. M., & Gardner, P. M. (July 01, 2013). A Stream-Based Methane Monitoring Approach for Evaluating Groundwater Impacts Associated with Unconventional Gas Development. *Groundwater, 51*, 4, 511-524.

Hershey AE, Beaty S, Fortino K, Kelly S, Keyse M, Luecke C, O'Brien WJ, Whalen SC. 2006. Stable isotope signatures of benthic invertebrates in arctic lakes indicate limited coupling to pelagic production. Limnol Oceanogr. 51:177–188.

Hershey AE, Beaty S, Fortino K, Kelly S, Keyse M, Luecke C, O'Brien WJ. 2005. δ13C signatures of chironomids in arctic lakes: role and direction of benthic-pelagic coupling. Verh Internat Verein Limnol. 29(1):292–295.

Houghton, J. T., & Intergovernmental Panel on Climate Change. (2001). Climate change 2001: The scientific basis: contribution of Working Group I to the third assessment report of the Intergovernmental Panel on Climate Change. Cambridge: Cambridge University Press.

Houghtona, D. C. (March 01, 2004). Utility of Caddisflies (Insecta: Trichoptera) as Indicators of Habitat Disturbance in Minnesota. Journal of Freshwater Ecology, 19, 1, 97-108.

Huryn, A. D., & Wallace, J. B. (January 01, 2000). Life History and Production of Stream Insects. *Annual Review of Entomology, 45,* 1.

Ings, N. L., Hildrew, A. G., & Grey, J. (2010). Gardening by the psychomyiid caddisfly Tinodes waeneri: evidence from stable isotopes. Oecologia, 163(1), 127-139.

JMP®, Version 10. SAS Institute Inc., Cary, NC, 1989-2012.

Jones, J. B. & Mulholland, P. J. (January 01, 1998). Methane input and evasion in a hardwood forest stream: Effects of subsurface flow from shallow and deep pathways. *Limnology and Oceanography, 43,* 6, 1243.

Jones RI, Grey J. 2011. Biogenic methane in freshwater food webs. Freshwater Biol. 56:213–229.

Jones RI, Carter CE, Kelly A, Ward S, Kelly DJ, Grey J. 2008. Widespread contribution of methane-cycle bacteria to the diets of lake profundal chironomid larvae. Ecology. 89:857–864.

Kajan, R., and Frenzel, P. (February 01, 1999). The effect of chironomid larvae on production, oxidation and fluxes of methane in a flooded rice soil. Fems Microbiology Ecology, 28, 2, 121-129.

Loudon, C., and Alstadd, D. N. 1992. Architectural plasticity in net construction by individual caddisfly larvae (Trichoptera: Hydropsychidae). Can. J. Zool. 70: 1 166 - 1 172.

Mackay, R.J., and T.F. Waters. 1986. Effects of small impoundments on hydropsychid caddisfly production in Valley Creek, Minnesota. Ecology 67:1680-1686.

Merritt R.W. and Cummins K.W. (2008) *An Introduction to the Aquatic Insects of North America*. (4th ed.). Dubuque: Kendall/Hunt Publishing.

Meyer JL. 1980. Dynamics of phosphorus and organic matter during leaf decomposition in a forest stream. Oikos 34: 44-53.

Parker, C.R., and J.R. Voshell, Jr. 1983. Production of filter-feeding Trichoptera in an impounded and a free-flowing river. Canadian Journal of Zoology 61:70-87.

Parker, C.R., and J.R. Voshell, Jr. 1982. Life histories of some filter-feeding Trichoptera in Virginia. Canadian Journal of Zoology 60:1732-1742.

Philipson, G. N., and Moorhouse, B. H. S. (December 01, 1974). Observations on ventilatory and net-spinning activities of larvae of the genus Hydropsyche Pictet (Trichoptera, Hydropsychidae) under experimental conditions. Freshwater Biology, 4, 6, 525-533.

Schaefer EF. 1997. A DNA assay to detect the toxic dinoflagellate *Pfiesteria piscida*, and the application of a PCR based probe. MS Thesis University of North Carolina, Greensboro.

Shelley, F., Abdullahi, F., Grey, J., & Trimmer, M. (January 01, 2015). Microbial methane cycling in the bed of a chalk river: oxidation has the potential to match methanogenesis enhanced by warming. *Freshwater Biology*, *60*, 1, 150-160.

Shindell, D. T., Faluvegi, G., Koch, D. M., Schmidt, G. A., Unger, N., & Bauer, S. E. (October 29, 2009). Improved Attribution of Climate Forcing to Emissions. *Science*, *326*,5953, 716-718.

Smith, M. K. K., & NC Digital Online Collection of Knowledge and Scholarship (NCDOCKS). (2013). *Methanogens and methane oxidizing bacteria in forested, urban unrestored, and urban restored streams*. Greensboro, N.C: University of North Carolina at Greensboro.

Trimmer, M., Hildrew, A. G., Jackson, M. C., Pretty, J. L., & Grey, J. (September 01, 2009). Evidence for the role methane-derived carbon in a free-flowing, lowland river food web. *Limnology & Oceanography*, *54*, 5.

US Geological Survey. (2015). US Department of the Interior. http://nwis.waterdata.usgs.gov/nc/nwis/dvstat? Accessed 18 April 2015.

Valentine DL, Reeburgh WS. 2000. New perspectives on anaerobic methane oxidation. Environ Microbiol. 2:477–484.

Wallace, J.B., and R.W. Merritt. 1980. Filter feeding ecology of aquatic insects. Annual Review of Entomology 25:103-132.

Wallace, J. B., J. R. Webster, and W. R. Woodall 1977. The role of filter-feeders in flowing waters. Arch. Hydrobiol. 79: 506-532.

Whalen SC. 2005. Biogeochemistry of methane exchange between natural wetlands and the atmosphere. Environ Eng Sci. 22:73–94.

Wright, A.-D. G., & Pimm, C. (November 01, 2003). Improved strategy for presumptive identification of methanogens using 16S riboprinting. Journal of Microbiological Methods,55, 2, 337-349.

APPENDIX A

TABLES AND FIGURES

Table 1. DNA Primers Used to Quantify Methanogen and Methane-Oxidizing Bacteria DNA Abundance.

Target Species	Target	Primers	Sequence	Reference
Methanogens	16S rRNA	Met 86 F	GCTCAGTAACACGTGG	Wright and Pimm 2003
		Met 1340R	GGTGTGTGCAAGGAG	
MOB	pmoA gene	A189gc	GGNGACTGGGACTTCTGG	Costello and Lindstrom 1999
		mb661	CCGGMGCAACGTCYTTACC	

Table 2. ANOVA of Seasonal DNA Abundance for Sampled Components with Tukey's Comparison.

Fall (11/9/2012) = Fa; Winter (3/8/2013) = Wi; Spring (6/5/2013) = Sp; Summer (9/20/2013) = Su; n.s = no significance.

2-Way ANOVA	Season analysis	Stream analysis	Date * Stream analysis
MET Foregut	Fa>Wi>Sp=Su; p < 0.0001	Reedy > NBC; p = 0.0002	n.s; p = 0.2149
MET Net	Fa=Wi=Su>Sp; p < 0.0001	NBC > Reedy; p = 0.0039	n.s; p = 0.1542
MET Retreat	Fa>Wi=Sp>Su; p < 0.0001	n.s; p = 0.9428	n.s; p = 0.9717
MET Sediment	Fa>Wi>Sp=Su; p < 0.0001	n.s; p = 0.2827	n.s; p = 0.2766
MET Seston	Fa=Wi=Su>Sp; p = 0.0088	n.s; p = 0.5941	n.s; p = 0.2132
MOB Foregut	Wi=Sp>Su=Fa; p < 0.0001	NBC > Reedy; p = 0.0116	Reedy Sp = NBC Fa, Wi, Sp > Su = Reedy Fa, Wi, Su; p = 0.0003
MOB Net	Wi=Su>Sp=Fa; p = 0.001	NBC > Reedy; p < 0.0001	NBC Wi > Sp, Su, Fa = Reedy Sp, Su, Fa, Wi; p = 0.0246
MOB Retreat	Fa=Sp>Wi=Su; p < 0.0001	NBC > Reedy; p = 0.0231	NBC Sp > Fa = Reedy Fa, Wi, Sp > Su = NBC Su, Wi; p < 0.0001
MOB Sediment	Wi>Fa=Sp>Su; p < 0.0001	Reedy > NBC; p < 0.0001	NBC Wi = Reedy Wi, Sp, Fa > Su = NBC Fa, Sp, Su; p < 0.0001
MOB Seston	Sp>Fa=Wi=Su; p < 0.0001	n.s; p = 0.0671	Reedy Sp > NBC Fa, Wi, Sp, Su = Reedy Fa, Wi, Su; p = 0.0081

1-Way ANOVA	Seasonal Mean DNA Concentration (ng/g), seston (ng/ml)								Statistical Analysis		
Reedy Fork MET	**11/9/2012**	**SE**	**3/8/2013**	**SE**	**6/5/2013**	**SE**	**9/20/2013**	**SE**	**F Ratio**	**p-value**	**Tukey's comparison**
Foregut	391.0	94.8	163.9	68.8	17.4	5.2	7.4	2.6	26.4439	<0.0001	Fa=Wi>Sp=Su
Net	5.2	4.0	23.7	20.3	0.01	0.001	5.5	2.5	2.2824	0.2211	n.s
Retreat	96.1	12.2	14.5	10.5	2.3	1.1	0.01	0.004	24.2057	<0.0001	Fa>Wi=Sp, Sp=Su<Wi
Sediment	54.3	10.3	25.0	17.3	1.2	0.5	0.1	0.1	49.4025	<0.0001	Fa=Wi>Sp=Su
Seston	0.03	0.02	0.1	0.03	0.02	0.01	3.7 E-06	2.7 E-06	1.9978	0.1728	n.s
NBC MET											
Foregut	209.2	155.1	10.8	1.8	3.9	2.3	2.4	1.1	11.5873	0.0042	Fa>Wi=Sp=Su
Net	82.8	22.1	145.3	43.1	0.1	0.04	14.7	1.4	20.0938	0.0001	Fa=Wi=Su>Sp
Retreat	122.5	81.7	5.9	N/A	2.0	0.3	0.1	0.04	17.6777	0.0022	Fa>Wi=Sp=Su
Sediment	75.2	12.4	10.8	5.1	0.1	0.1	0.1	0.02	47.2548	<0.0001	Fa>Wi>Sp=Su
Seston	0.1	0.03	0.1	0.02	0.005	0.003	0.002	0.001	7.3608	0.0085	Fa=Wi>Sp=Su
Reedy Fork MOB											
Foregut	1.9	0.5	9.1	1.3	57.1	18.8	4.6	2.4	20.0282	<0.0001	Sp>Wi=Su, Su=Fa<Wi
Net	0.01	N/A	1.9	0.7	0.3	0.1	2.5	1.2	2.3747	0.1865	n.s
Retreat	13.5	2.0	13.0	7.0	4.2	1.1	0.04	0.02	12.6888	0.0004	Fa=Wi=Sp>Su
Sediment	16.6	2.7	24.3	2.8	21.9	12.6	0.3	0.1	41.9382	<0.0001	Fa=Wi=Sp>Su
Seston	0.0001	0.00003	0.004	0.002	0.1	0.04	0.002	N/A	5.6074	0.0281	Sp>Fa=Su=Wi
NBC MOB											
Foregut	14.1	2.9	32.4	10.7	15.7	4.6	10.2	3.6	2.3122	0.1176	n.s
Net	8.7	2.8	357.7	123.4	3.3	1.3	40.0	19.5	14.5486	0.0003	Wi>Sp=Su=Fa
Retreat	50.4	41.4	0.2	0.1	137.1	28.1	1.0	0.2	37.764	<0.0001	Sp>Fa>Su=Wi
Sediment	1.8	1.2	31.0	8.7	0.7	0.3	0.2	0.1	43.490	<0.0001	Wi>Sp=Su=Fa
Seston	0.001	0.0004	0.002	0.001	0.03	0.01	0.003	0.001	9.750	0.001	Sp>Fa=Su=Wi

Table 3. Summary ANOVA's of Foregut and Net MOB DNA Abundance with Tukey's Comparison.

Stream sites include: NBC = North Buffalo Creek, Reedy = Reedy Fork. Treatments include: SB = Sediment Bubbled, NSB = No Sediment Bubbled, NSNB No Sediment No Bubbled. Caddisflies include H = Hydropsyche, C = Cheumatopsyche. Tukey's tests were performed for the statistical analysis for each ANOVA, except for stream and caddisfly where t-tests were performed because of the lack of degrees of freedom to perform a Tukey's test.

Analysis	F-ratio	n	df	p-value	Statistical Analysis
MOB Net 2 Way ANOVA					
Stream	11.9455	2	1	0.001	NBC > Reedy
Treatment	17.2331	3	2	<0.0001	SB > NSB > NSNB
Stream * Treatment	3.8209	3	2	0.027	NBC SB = NBC NSB = Reedy SB > Reedy NSB = NBC NSNB = Reedy NSNB
MOB Net 1 Way ANOVA by Stream					
Treatment, NBC	5.3643	3	2	0.0096	SB = NSB > NSNB
Treatment, Reedy	19.9435	3	2	<.0001	SB > NSB = NSNB
MOB Gut 3 Way ANOVA					
Stream	6.4291	2	1	0.0124	NBC > Reedy
Caddisfly	0.1806	2	1	0.6716	n.s
Treatment	12.7907	3	2	<.0001	SB > NSB > NSNB
Stream * Caddisfly	0.0723	2	1	0.7885	n.s
Caddisfly * Treatment	0.2234	3	2	0.8001	H SB = C SB = C NSB = H NSB, C NSB = H NSB = C NSNB = H NSNB < H SB = C SB
Stream * Treatment	1.97	3	2	0.1435	NBC SB = NBC NSB = Reedy SB > Reedy NSB = NBC NSNB = Reedy NSNB
Stream * Caddisfly * Treatment	0.5621	3	2	0.5714	NBC C SB > Reedy H NSB= Reedy C NSNB, All other comparisons are equal
MOB Gut 2 Way ANOVA					
Stream	6.6297	2	1	0.0111	NBC > Reedy
Treatment	13.1899	3	2	<.0001	SB > NSB > NSNB
Stream * Treatment	2.0315	3	2	0.135	NBC SB = NBC NSB = Reedy SB > Reedy NSB = NBC NSNB = Reedy NSNB
MOB Gut 1 Way ANOVA by Stream					
Treatment, NBC	5.9888	3	2	0.004	SB = NSB > NSNB
Treatment, Reedy	10.7677	3	2	<.0001	SB > NSB = NSNB

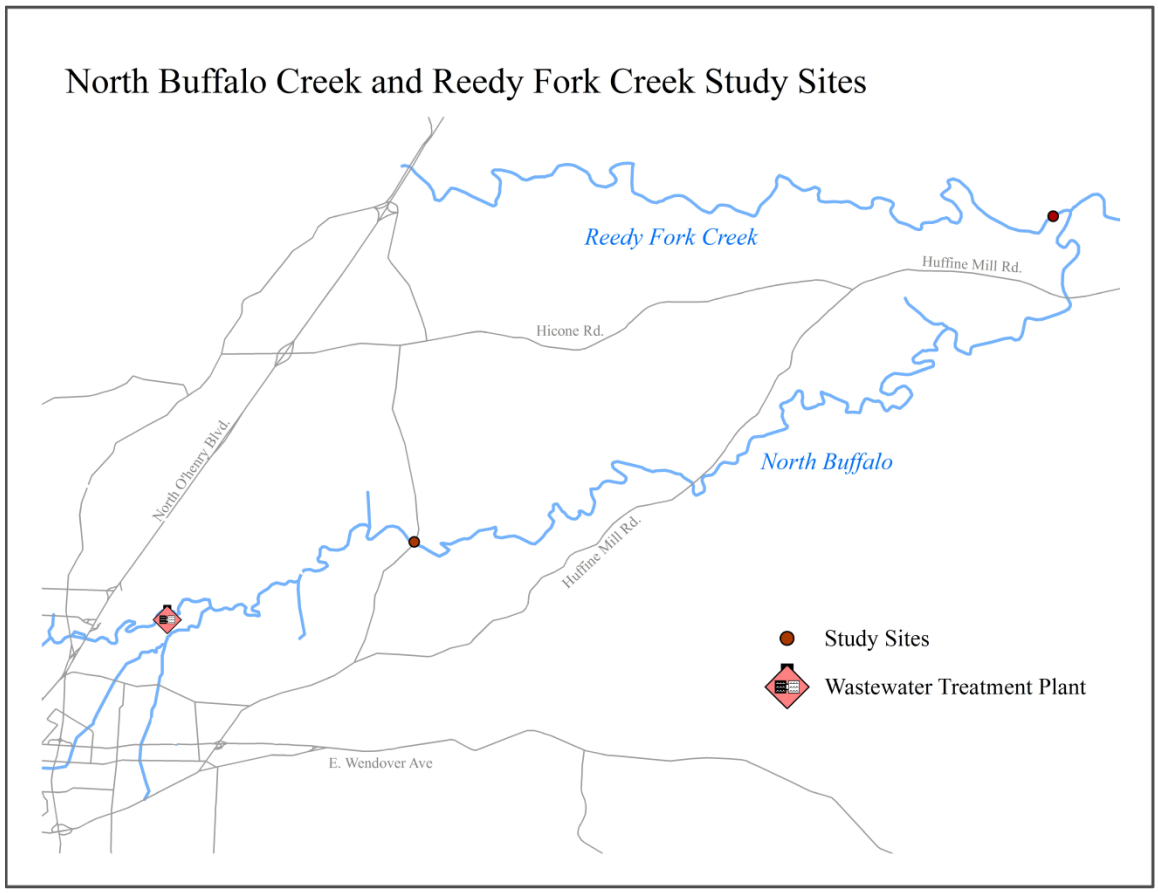

Figure 1. Field Sampling Sites.

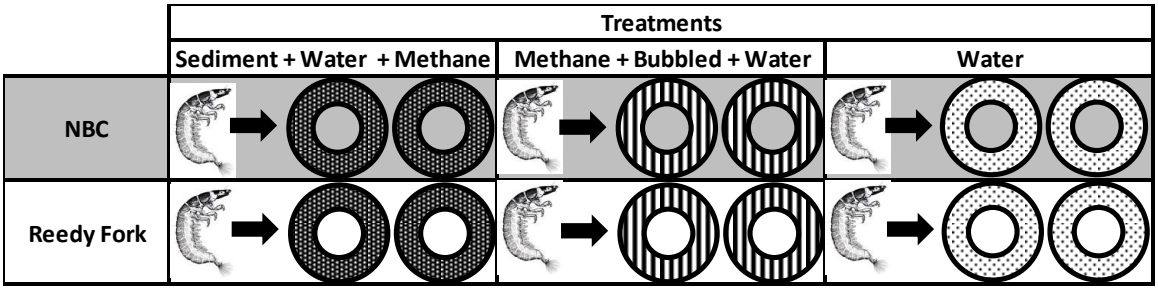

Figure 2. Artificial Stream Experimental Setup.

Three different treatments assessed with two different sources of water and sediment per treatment (NBC and Reedy Fork). A total of six artificial stream reservoirs were created to reflect the six treatments shown. Two circulating artificial streams were placed into each stream reservoir to evaluate possible differences between the artificial streams. Both genera of caddisflies (*Cheumatopsyche* and *Hydropsyche*) were introduced into each artificial stream.

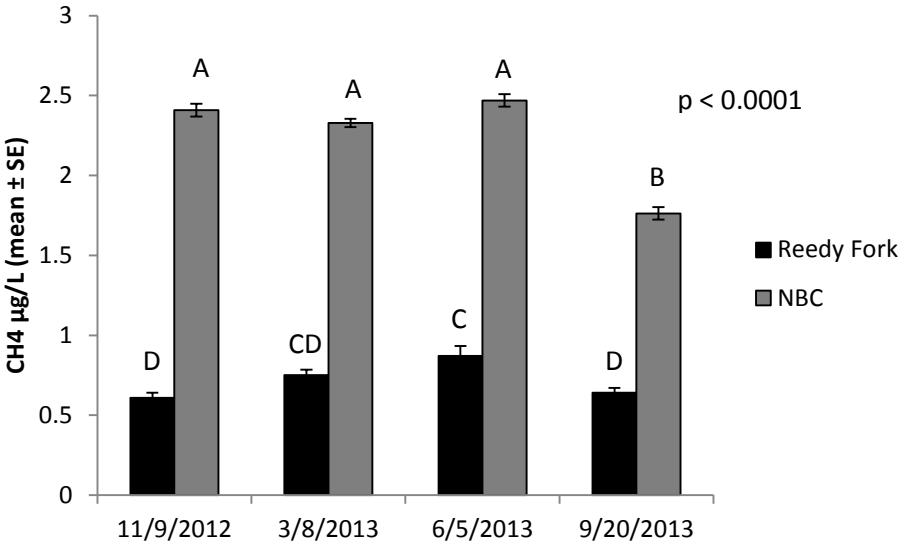

Figure 3. Methane Concentration at Study Sites ± SE.

Different letters above each bar indicate significant differences in the mean methane concentration based on a Tukey's test.

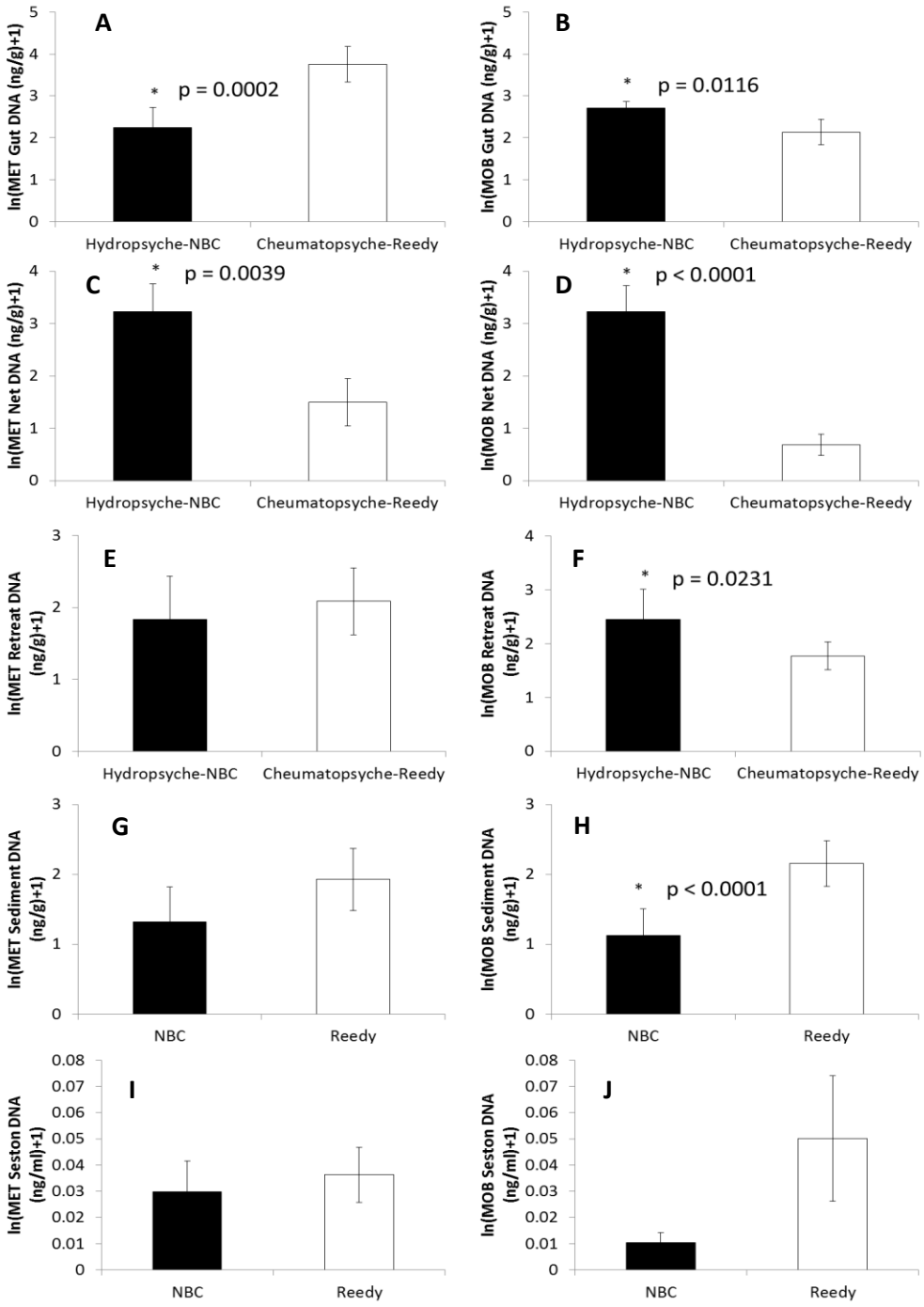

Figure 4. DNA Abundance for Sampled Components by Genus or Stream ± SE.

Values on the graph compare methanogens (left column) and MOB (right column) for each sampled component. A & B: foreguts, C & D: nets, E & F: retreats, G & H: sediment, I & J: seston.

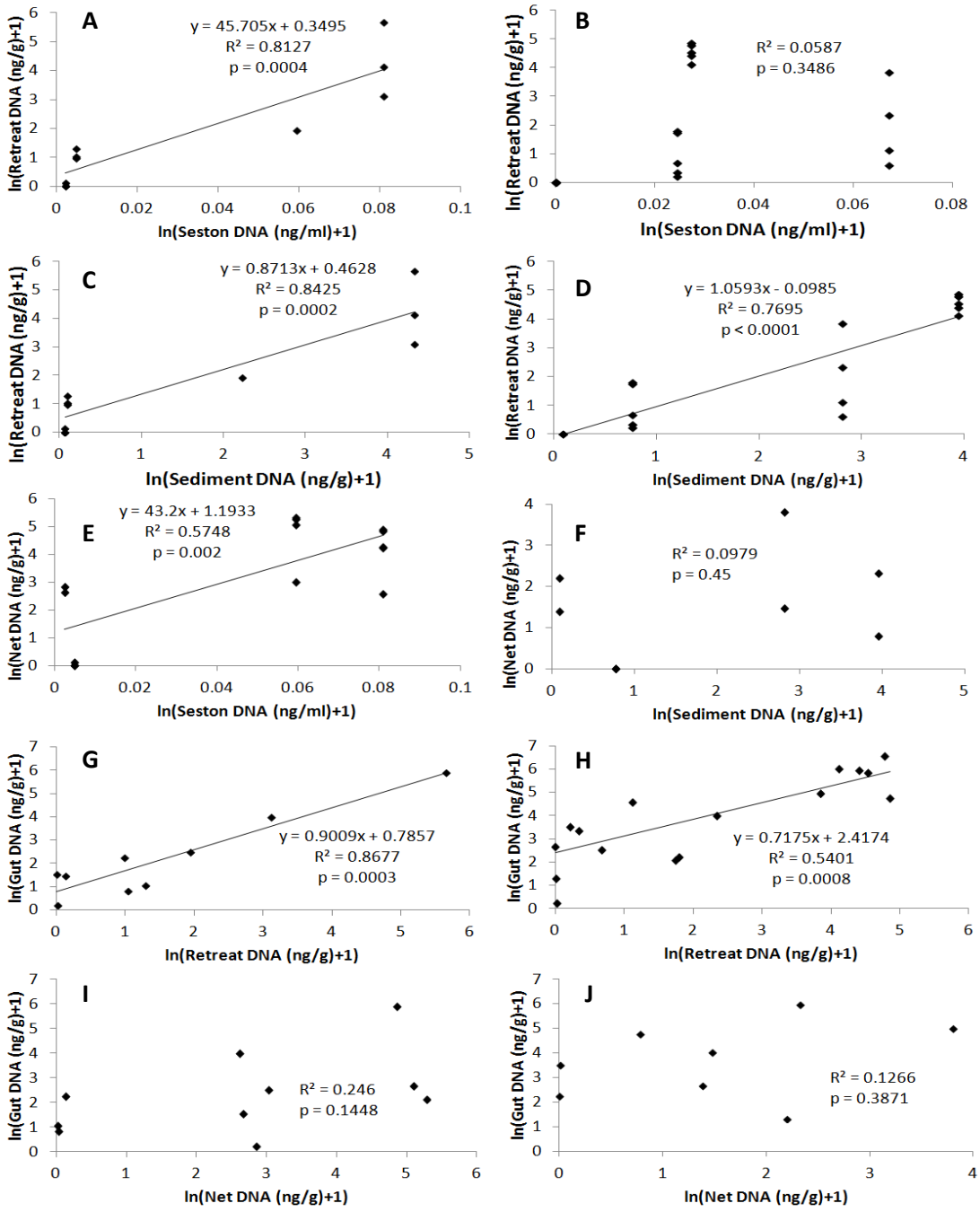

Figure 5. Stream Study Sites and Caddisfly Methanogen Regression Analysis.

Values on the graph evaluate responses through regression analysis of NBC *Hydropsyche* (left column) and Reedy Fork *Cheumatopsyche* (right column) for methanogen sampled component. Panel A & B: retreat vs. seston; C & D: retreat vs. sediment; E: nets vs. seston; F: nets vs. sediment; G & H: foregut vs. retreat; I & J: foregut vs. net.

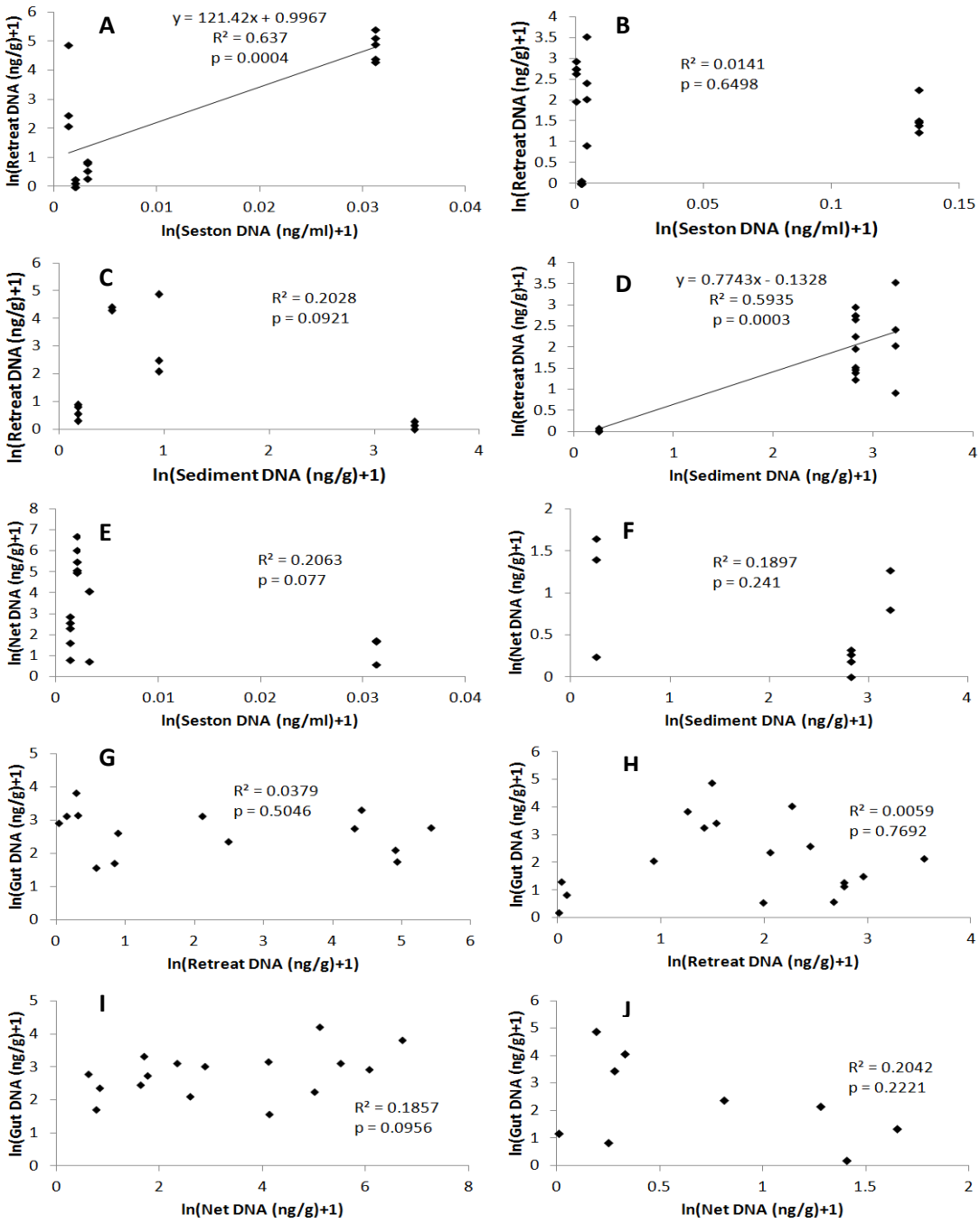

Figure 6. Stream Study Sites and Caddisfly MOB Regression Analysis.

Values on the graph evaluate responses through regression analysis of NBC *Hydropsyche* (left column) and Reedy Fork *Cheumatopsyche* (right column) for MOB sampled components. Panel A & B: retreat vs. seston; C & D: retreat vs. sediment; E: net vs. seston; F: net vs. sediment; G & H: foregut vs. retreat; and I & J: foregut vs. net.

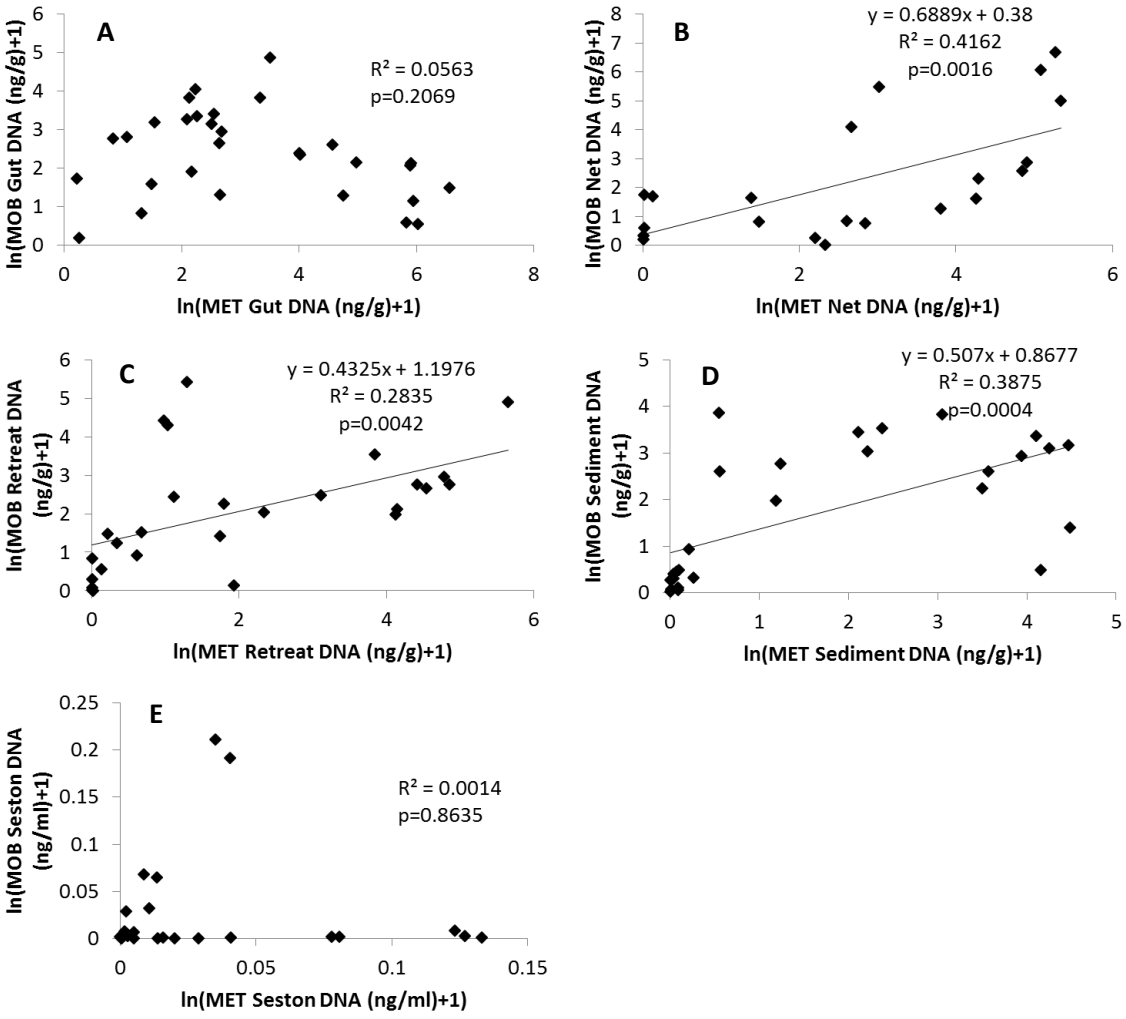

Figure 7. Stream and Caddisfly Methane-Oxidizing Bacteria Response to Methanogens.

Regression analysis of MOB DNA abundance vs. Methanogen DNA abundance sampled from: A) Foregut contents, B) Caddisfly nets, C) Caddisfly retreats, D) Stream sediment, and E) Stream seston. Foregut, net, and retreat graphs included pooled data for *Hydropsyche* and *Cheumatopsyche*, while sediment and seston graphs included pooled data for NBC and Reedy Fork.

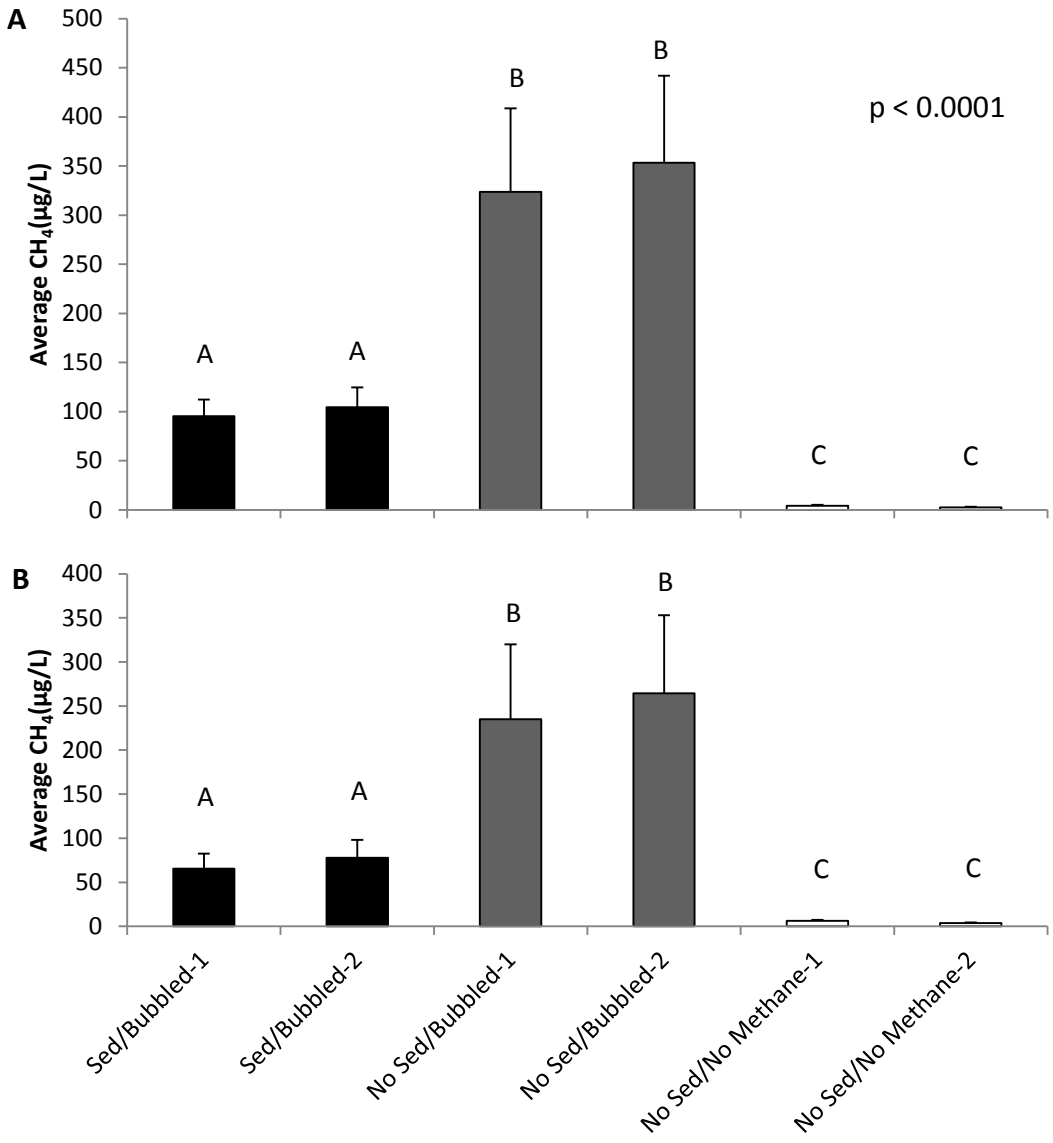

Figure 8. Mean Artificial Stream Methane Concentration throughout Experiment ± SE.

Three different treatments are shown for each stream source (Panel A- North Buffalo Creek or Panel B- Reedy Fork). Both artificial streams were sampled for each treatment type and are designated as such by either a one or two following their treatment names. Different letters above each bar indicate significant differences in the mean methane concentration based on a Tukey's test.

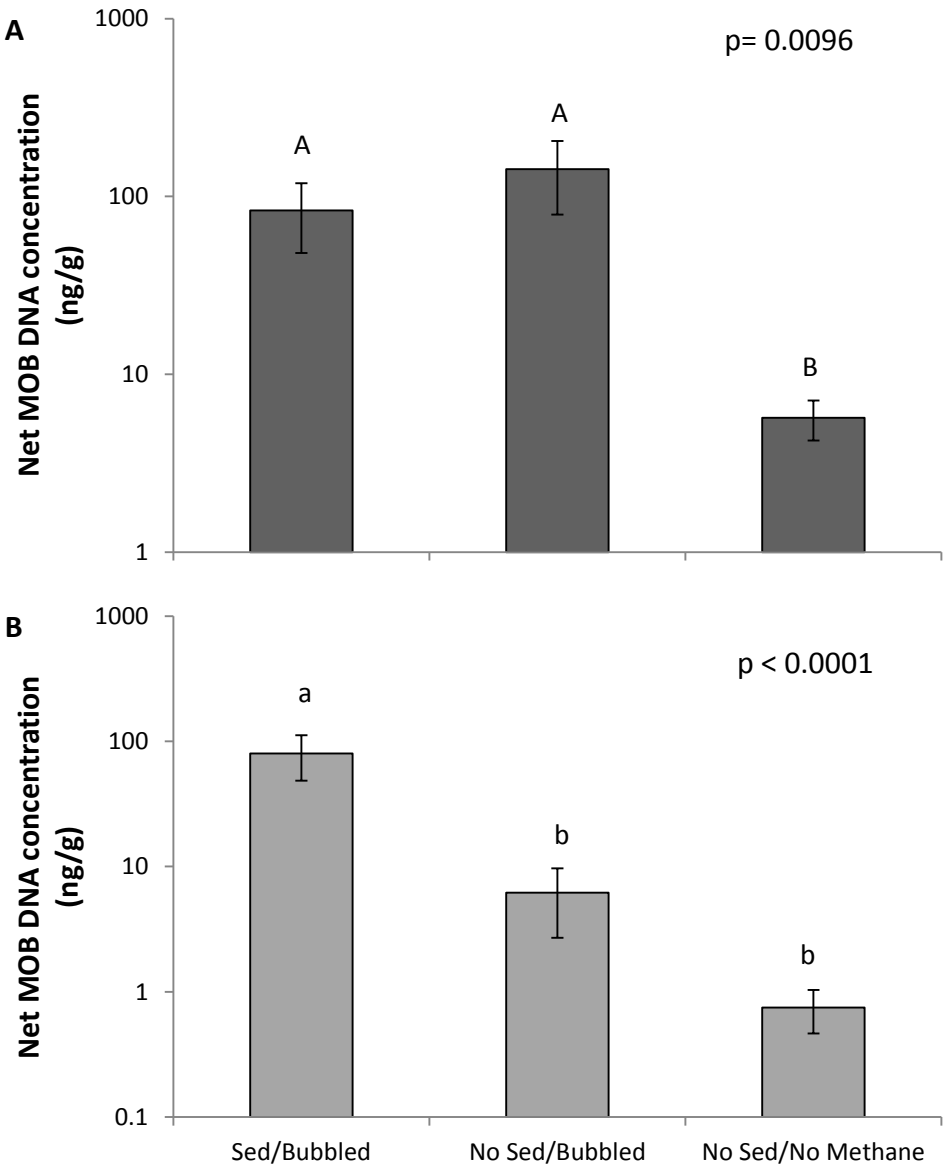

Figure 9. Mean Net Methane-Oxidizing Bacteria DNA Abundance ± SE.

Caddisfly genera were pooled for each graph. Panel A is a treatment comparison of net DNA of caddisflies in North Buffalo Creek and Panel B is a treatment comparison of net DNA of caddisflies in Reedy Fork. Different letters above each bar indicate significant differences in the mean DNA abundance based on a Tukey's test. Statistics including the Tukey's test were run on log transformed data.

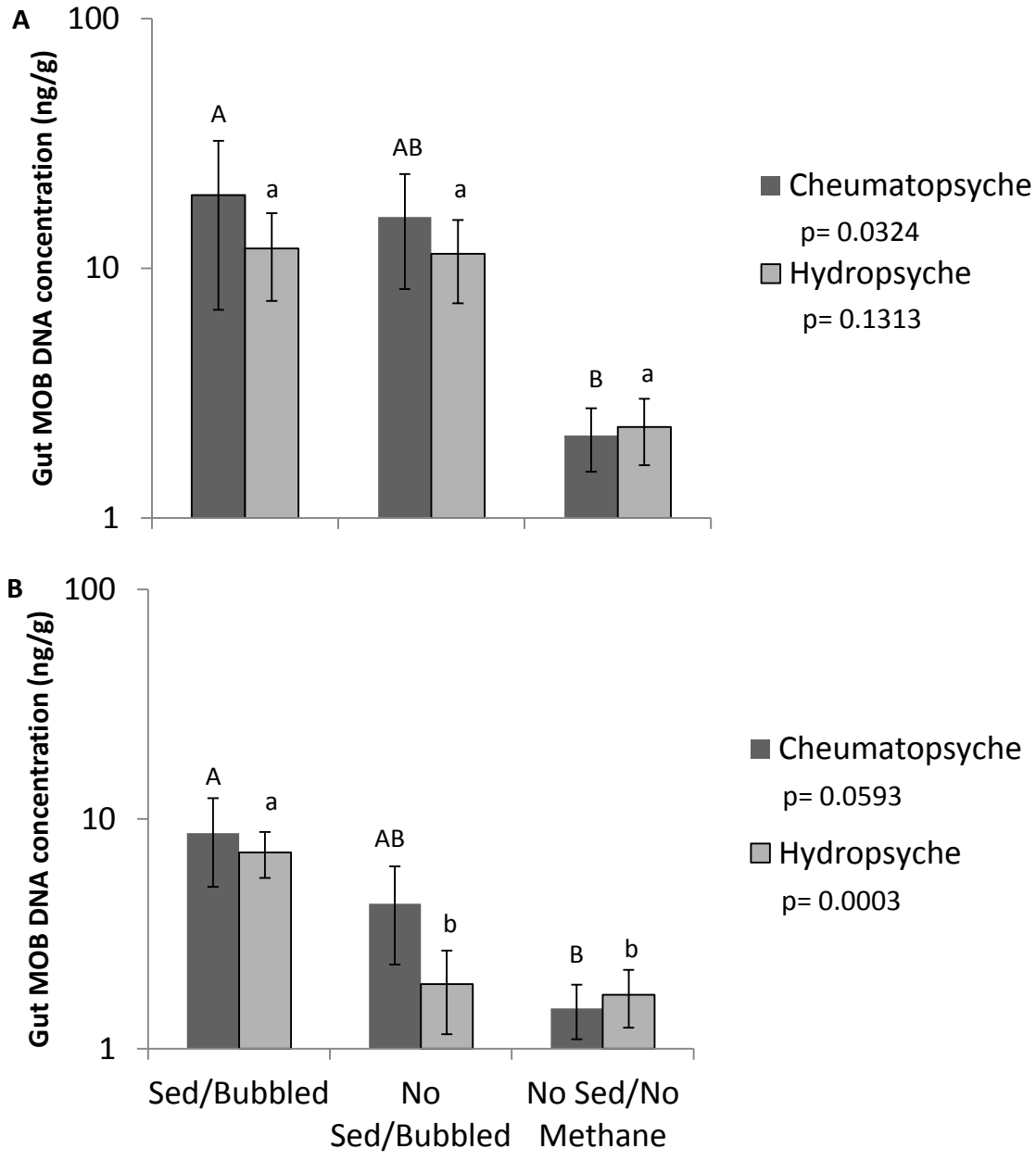

Figure 10. Mean Foregut Methane-Oxidizing Bacteria DNA Abundance ± SE.

Graphs are treatment comparisons of foregut DNA by genera and stream type. Panel A compares foregut abundance between NBC caddisflies and Panel B compares foregut abundance between Reedy Fork caddisflies. Different letters above each bar indicate significant differences in the mean DNA abundance based on a Tukey's test. All statistics including the Tukey's test were run on log transformed data. P-values for corresponding Tukey's tests are listed under the corresponding legend.

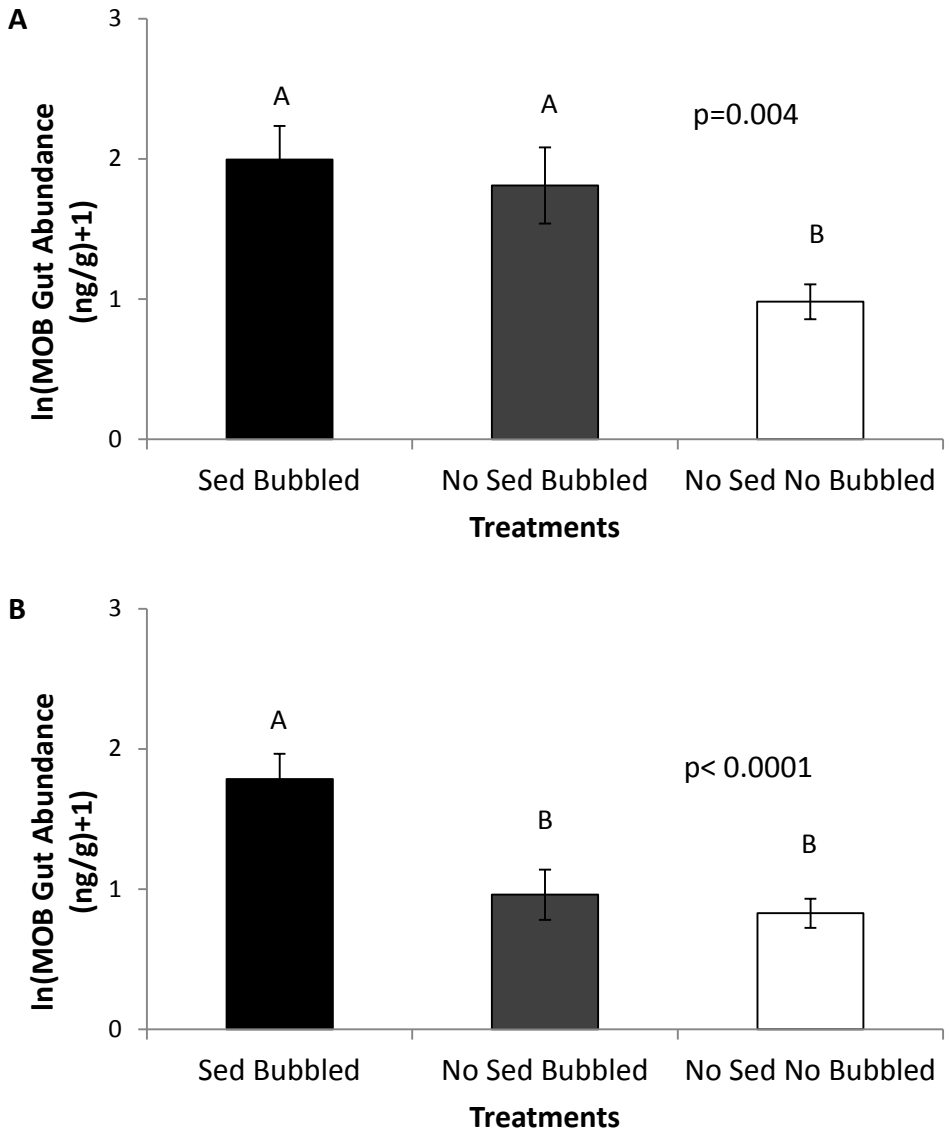

Figure 11. Mean Methane-Oxidizing Bacteria Foregut DNA Abundance ± SE by Stream.

Caddisfly genera were pooled for both graphs. All values had 1 added and were log transformed as necessary. Graph A is a treatment comparison of foregut DNA of caddisflies in North Buffalo Creek and Graph B is a treatment comparison of foregut DNA of caddisflies in Reedy Fork. Different letters above each bar indicate significant differences in the mean DNA abundance based on a Tukey's test.